

Med25 Is Required for RNA Polymerase II Recruitment to Specific Promoters, Thus Regulating Xenobiotic and Lipid Metabolism in Human Liver[∇]

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Hepatocyte nuclear factor 4 α (HNF4 α) controls the expression of many critical metabolic pathways, and the Mediator complex occupies a central role in recruiting RNA polymerase II (Pol II) to these gene promoters. An impaired transcriptional HNF4 α network in human liver is responsible for many pathological conditions, such as altered drug metabolism, fatty liver, and diabetes. Here, we report that Med25, an associated member of the Mediator complex, is required for the association of HNF4 α with Mediator, its several cofactors, and RNA Pol II. Further, increases and decreases in endogenous Med25 levels are reflected in the composition of the transcriptional complex, Pol II recruitment, and the expression of HNF4 α -bound target genes. A novel feature of Med25 is that it imparts “selectivity.” Med25 affects only a significant subset of HNF4 α target genes that selectively regulate drug and lipid metabolism. These results define a role for Med25 and the Mediator complex in the regulation of xenobiotic metabolism and lipid homeostasis.

The liver is the central organ that controls/regulates such metabolic processes as lipid metabolism (lipogenesis and fatty acid β -oxidation [FAO]), energy homeostasis (glycolysis and gluconeogenesis), bile acid synthesis, and drug metabolism (4, 48, 49, 57). Metabolism in the liver is governed by a highly dynamic transcriptional regulatory network whose key receptors may be classical (glucocorticoid), adopted (peroxisome proliferator activated receptor, farnesoid X receptor, liver specific X receptor, and constitutively active receptor/pregnane X receptor), or orphan (hepatocyte nuclear factor 4 α [HNF4 α]) (6). HNF4 α , also known as NR2A1, is an important member of the orphan nuclear receptor superfamily (51, 53). When HNF4 α binds to DNA, it regulates the expression of both constitutive genes, such as apolipoprotein genes (37), *CYP7A1* and *CYP8B1* (25, 27), the FXII and XIIIIB genes (26), the proline oxidase gene (30), *CYP2D6* (5), *CYP2A6* (42), *UGT1A9* (1), *SULT1A1* (31), and the glucose-6-phosphatase (G6PC) and Pck1 genes (47), and also xenobiotic inducible genes, such as *CYP3A4* (56), *CYP2C9* (8), and *CYP2B6* (24). Malfunctions in the regulation of genes by HNF4 α are responsible for alterations in drug metabolism and metabolic disorders, such as atherosclerosis, diabetes, hemophilia, hypoxia, medium-chain acyl-coenzyme A (CoA) dehydrogenase (MCAD) deficiency, and ornithine transcarbamylase (OTC) deficiency (16, 22).

Like most nuclear receptors, HNF4 α displays a typical DNA binding domain (DBD), including a double zinc finger motif, a ligand binding domain (LBD), and two activation domains,

AF1 and AF2, located in the N- and C-terminal regions, respectively (21, 53). The DNA binding domain binds to a *cis*-acting element which optimally consists of a direct repeat of AGGTC A with a 1- or 2-nucleotide spacer (DR1/DR2) to which HNF4 α binds as a homodimer (52). The ligand binding domain is structurally homologous to other receptors of the retinoid receptor family, with a well-defined hydrophobic pocket capable of binding fatty acids (11, 21). Fatty acids have been long believed to act as ligands for many orphan receptors (23, 61); recently, Yuan and coworkers (64) showed that HNF4 α is selectively occupied by linoleic acid (LA; C18: omega6) in mammalian cells, although ligand occupancy was not necessary for transcriptional activity of HNF4 α , as shown by genome-wide profiling.

The AF2 motif of HNF4 α is responsible for binding to several groups of cofactors, such as the CBP/p300 class (10, 38), to members of the SRC family (59), including the histone acetyltransferase complex (62), to the NCOA6/PRIP-anchored ASCOM complex (17, 54), to the Med1-anchored Mediator complex (33, 38, 43), and to the energy sensor PGC-1 α (47). The diverse nature of these coactivators suggests that a variable functional complex not only controls the constitutive expression of genes but also directs specialized functions of the liver, such as fatty acid metabolism, drug metabolism, and energy homeostasis. These hepatic pathways are affected by the hormonal and environmental signals that induce metabolic syndrome, a combination of disorders that increase the risk of diabetes and cardiovascular diseases (13, 14, 19, 45, 65).

In this report, we identify a new coactivator of HNF4 α , Med25. We have demonstrated here that Med25 plays a vital role in modulating HNF4 α transcriptional activity by (i) converting the HNF4 α -bound transcriptional complex from the inactive to the active state, (ii) facilitating the recruitment of RNA polymerase II (Pol II) to the promoter site, and (iii)

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giving HNF4 α an exquisite specificity in controlling the genes that are responsible for lipid and drug metabolism, viz., the cytochrome P-450s.

MATERIALS AND METHODS

Cell lines and cell culture. HepG2 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin at 37°C under 5% CO₂. HEK293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS. Primary human hepatocytes were obtained from CellDirect (Invitrogen, Durham, NC) and maintained in William's E medium supplemented with ITS (insulin, human transferrin, sodium selenite, bovine serum albumin, and linoleic acid in Earle's balanced salt solution; Sigma), HEPES, L-glutamine, and 100 nM dexamethasone.

Plasmids and adenovirus (Ad) construction. Full-length Med25 was cloned from a Mammalian Gene Collection (MGC) clone, BCO65297, into the pcDNA3.1, p3XFLAG-CMV-7.1, and pEGFP-N1 vectors. Deletion constructs of Med25 were generated in the pGEX4T1 and pACT vectors. Mutation constructs were prepared using a site-directed mutagenesis kit (Stratagene) from the wild-type constructs. All constructs were sequence verified.

For adenoviral production, Flag-Med25, HNF4 α , and CAR were cloned in a pShuttle vector and recombined with an AdEasy vector backbone. Short hairpin RNAs (shRNAs) were identified using Genescript's target finder and cloned into pRNAT-H1.1/Adeno vector. The viruses were produced and amplified in AD293 cells using standard procedures. Viral particles were purified on a CsCl gradient and stored in sucrose buffer.

Yeast and mammalian two-hybrid screening and assay. ProNet Technologies automated two-hybrid screening was performed by Myraid Genetics (Salt Lake City, UT) as described previously (15). A liver cDNA library was used to screen proteins that interact with HNF4 α . To map the interaction domains between Med25 and HNF4 α , deletion derivatives of Med25 were fused to VP16 AD in a pACT vector. The ligand binding domain of HNF4 α was fused to the GAL4 DBD in a pBind vector. The promoter luciferase reporter 9XGAL4 UAS was employed to assay the interaction in HepG2 cells. Promega's Dual-Glo System was used to measure the luciferase activity.

GST pulldown assays. Glutathione S-transferase (GST) fusion proteins of nuclear receptors (CAR, PXR, and HNF4 α) were expressed in *Escherichia coli* BL-21, and Med25 fragments were purified on glutathione (GSH)-Sepharose beads (Amersham). It should be noted that the endogenous ligand of HNF4 α (linoleic acid) is not present in bacteria, but crystallographic studies of recombinant HNF4 α indicate that bacterial fatty acids occupy the ligand binding pocket (11). The indicated full-length proteins were translated *in vitro* in rabbit reticulocyte lysate (TNT Quick coupled transcription and translation system; Promega) with radiolabeled [³⁵S]methionine (MP Biomedicals). Expression and purification of recombinant GST fusion proteins and interaction assays were described previously (54).

Nuclear extract preparation, immunoblot analysis, and protein identification by LC-MS-MS. Nuclear extracts were prepared from HepG2 cells and primary human hepatocytes with reduced or increased Med25 as described before (12). Nuclear extracts from both of the preparations were subjected to immunoprecipitation (IP) with rabbit anti-HNF4 α antibody (Ab) (sc8987)-coupled agarose beads using a coimmunoprecipitation kit (Pierce). The bound proteins were eluted and silver stained using a SilverQuest silver-staining kit (Invitrogen) for visualization. Proteins were immunoblotted with the indicated antibodies. For mass spectrometry (MS) analysis, the protein bands from the Coomassie blue-stained gels were cut and digested with trypsin as described by Choi et al. (9). The resulting peptide digests were then analyzed by nano-liquid chromatography (nano-LC) electrospray ionization (ESI)-MS and tandem mass spectrometry (MS-MS) on an Agilent XCT Ultra ion trap mass spectrometer. The data were processed and searched against the NCBI nonredundant database as previously described (9).

Transfections and confocal microscopy. Transfections in HepG2 and HEK293 cells were performed with the indicated plasmids using Lipofectamine 2000 (Invitrogen). For primary human hepatocytes, transfections were performed on freshly isolated human hepatocytes without overlay using Effectene transfection reagent (Qiagen) according to the manufacturer's procedures.

For colocalization studies, HEK293 cells were transfected on chamber slides with enhanced green fluorescent protein (EGFP)-Med25 and the indicated cDNA constructs. The slides were processed for immunostaining, and the proteins were localized with a Zeiss LSM-510 UV confocal microscope.

Promoter assay and gene induction. HepG2 cells or primary human hepatocytes were seeded in 24-well plates at a density of 2.5×10^5 cells per well. After 24 h, the cells were transiently transfected and infected sequentially. Forty-eight hours after transfection, cells were harvested and assayed with a Dual-Glo luciferase assay system (Promega) using a LUMIstat OPTIMA. Gene expression analyses were performed by TaqMan assay with an ABI 7900 while the RNA was isolated using standard Qiagen procedures. The relative quantities were calculated by the $2^{-\Delta\Delta CT}$ method.

EMSAs and ChIP analysis. Electrophoretic mobility shift assays (EMSAs) were performed as described before, using a double-stranded ³²P-labeled oligonucleotide containing the bp -150 HNF4 α response element of the CYP2C9 promoter (8). Conventional chromatin immunoprecipitation (ChIP) analysis was performed on HepG2 cells treated with the indicated adenovirus as described before (54), while for primary human hepatocytes, ChIP was performed according to the Magnify ChIP procedure (Invitrogen, CA). Quantitative PCR (qPCR) was performed using Power SYBR green master mix (Applied Biosystems).

Fatty acid oxidation assay. Primary human hepatocyte homogenates were prepared in a homogenization buffer containing 300 mM mannitol, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2). The homogenates were centrifuged at $1,000 \times g$ for 1 min, and supernatants were used for the acyl-CoA oxidase activity as described previously (41).

RESULTS

Identification of Med25 as a novel interactor of hepatocyte nuclear factor 4 α . We identified Med25 as an HNF4 α ligand binding domain interacting protein in a yeast two-hybrid screen with a human liver cDNA library. Med25 is also known as p78, PTOV2, ACID1, or ARC92 (2, 3, 39, 58, 63). Two clones of Med25 that code for amino acids 583 to 747 and 624 to 702, respectively, were isolated; both clones include the NR binding motif, LXXLL. The function of Med25 is indicated by the conserved domain database search described earlier (32), showing a distinct VWA domain, a PTOV domain between two SD domains, and an NR-interacting domain. It appears that Med25 functions to efficiently coordinate the NR-based transcriptional activation. Only one other Mediator complex protein, Med1, has been reported to bind to HNF4 α (38).

To confirm the interaction between Med25 and HNF4 α , we tested the interaction between Med25 and HNF4 α as well as that between CAR and PXR, another pair of key regulators of hepatic xenobiotic metabolism. Our data showed that Med25 exhibited a strong interaction with HNF4 α but failed to interact with CAR and PXR (Fig. 1A, top). PGC-1 α , a known cofactor for the HNF4 α ligand binding domain, interacted with both CAR and HNF4 α (Fig. 1A, bottom).

To identify the interacting region between Med25 and HNF4 α , we generated a series of constructs (Fig. 1B) expressing Med25 domains as GST fusion proteins. Med25 specifically interacted with HNF4 α , and the interaction was mediated through the LXXLL motif (Fig. 1C). The interaction was lost when the LXXLL motif was mutated to LXXAA. HNF4 α -Med25 interactions were assayed with no additional ligand, but Med25 domains showed a classical ligand-dependent interaction with retinoic acid receptor (RAR) (Fig. 1C), in agreement with an earlier report (32). In addition, the partial domains failed to interact with CAR and PXR in the presence or absence of their respective ligands.

To further assess the interaction strength and specificity between Med25 and HNF4 α *in vivo*, we employed a mammalian two-hybrid assay, in which Med25 domains representing the N-terminal (VWA region), the middle (PTOV region), and the C-terminal (NR box) regions were assayed with the ligand binding domain of HNF4 α . Med25 domain III specifically in-

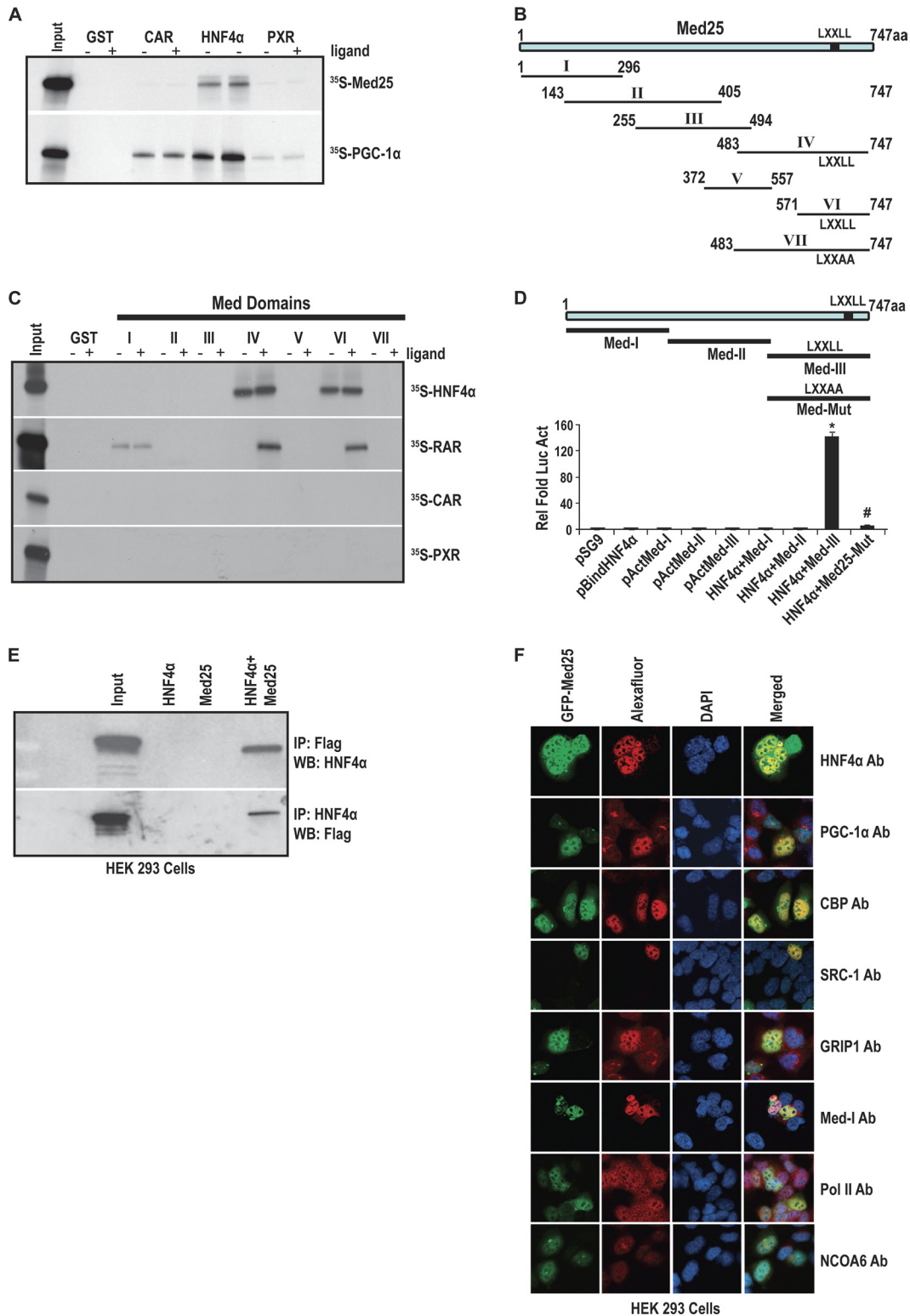


FIG. 1. Med25 interacts with HNF4 α and cofactors *in vitro*. (A) Med25 interacts with HNF4 α *in vitro*. Nuclear receptors CAR, PXR, and HNF4 α were expressed as GST fusion proteins and incubated with full-length *in vitro*-translated ³⁵S-labeled Med25 (top panel) and PGC-1 α (lower panel). CAR was incubated with Med25 and PGC-1 α in the presence of 100 nM CAR ligand CITCO, and PXR was incubated with Med25 and PGC-1 α in the presence of 10 μ M PXR ligand rifampin. HNF4 α was incubated with Med25 and PGC-1 α without exogenous ligand. DMSO, dimethyl sulfoxide. (B) Schematic representation of Med25 along with generated domains for interaction mapping. The numbers indicate the amino acid (aa) residues, and the length of the recombinant protein fragment, and LXXLL denotes the NR-interacting motif. (C) Med25 interacts with HNF4 α through the LXXLL domain. GST-Med25 domains were incubated with the indicated *in vitro*-translated ³⁵S-labeled nuclear receptors in the presence and absence of ligands (for CAR, CITCO; for PXR, rifampin; for RAR, *trans*-retinoic acid; and for HNF4 α , no

teracted with the HNF4 α LBD as shown (Fig. 1D). The mutated construct expressing LXXAA instead of LXXLL did not show any appreciable interaction. Consistent with the GST pulldown assay, no interactions were found when Med25 domains were assayed with the CAR or PXR xenobiotic sensing nuclear receptor (data not shown). These results confirm that Med25 associates with HNF4 α but not with CAR or PXR.

HNF4 α binds with Med25 and other coactivators *in vivo* and *in vitro*. To confirm the interaction between HNF4 α and Med25 *in vivo*, we performed a reciprocal immunoprecipitation assay with HEK293 cells. Since the expression levels of HNF4 α and Med25 are negligible in HEK293 cells, HNF4 α and Flag-tagged Med25 were ectopically expressed. Med25 was indeed shown to interact with HNF4 α *in vivo* via IP with Flag antibody and subsequent immunoblotting of the immunoprecipitates with HNF4 α antibody (Fig. 1E, top) and via a reciprocal basis IP with HNF4 α antibody and immunoblotting with anti-Flag antibody (Fig. 1E, bottom).

To identify HNF4 α binding proteins, we extracted nuclear proteins from HepG2 cells that had been infected with adenovirus coding for HNF4 α and Flag-tagged Med25. Although HNF4 α is expressed in HepG2 cells, ectopic expression of HNF4 α further induces HNF4 α target genes, e.g., the *CYP2C* genes (29). Because a commercial antibody for Med25 was not available, we tracked the expression of Med25 by a Flag-tagged recombinant protein. The HepG2 nuclear extract was coimmunoprecipitated with IgG or anti-HNF4 α antibody. When the bound proteins were separated on a 4 to 20% SDS-PAGE gel (see Fig. S2A at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>), silver stained, and subjected to mass spectral identification, a variety of cofactors were immunoprecipitated with anti-HNF4 α antibody and very few proteins with IgG (control). Some of the notable proteins identified by mass spectrometry and confirmed by immunoblotting (see Fig. S2B at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>) include Med1, Med8, CBP, and NCOA6. The entire list of proteins identified is given in Table S-I at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>. Interestingly, the proteins that were coimmunoprecipitated with HNF4 α represent the majority of the known subcomplexes, i.e., the chromatin-modifying group (CBP and SRC family), the Mediator group (Med1 and Med8), the ASCOM complex (NCOA6), the RNA polymerase II (Pol II) complex, and associated proteins such as PGC-1 α , in addition to HNF4 α and Med25. The identification of proteins representing all the subcomplexes suggests that HNF4 α binds to these proteins in a

functional transcriptional complex such as the preinitiation complex (PIC).

As the conserved-domain database search revealed four different domains in Med25, we hypothesized that these domains could play a role in different cofactor associations. When we examined fragments of Med25 containing single domains, we found that Med25 interacted with HNF4 α through the LXXLL motif in fragments IV and VI (see Fig. S2C at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). PGC-1 α , one of the previously known cofactors, bound to fragment IV, fragment V, and fragment VII, but not to fragment VI, suggesting that PGC-1 α interacts in the C-terminal region of Med25 but that binding is not dependent on the LXXLL motif. SRC-1/NCOA1 and GRIP-1/SRC-2/NCOA2 (which are part of the CBP binding complex involved in chromatin modifications and HAT-containing enzymes) were found to bind exclusively with Med25 fragment I, which codes for the VWA domain. Interestingly, although NCOA6 coimmunoprecipitated with HNF4 α and is known to be an interacting cofactor of this receptor, it did not interact with Med25 directly (data not shown). Among other proteins that bound to Med25, CBP was found to bind very strongly with Med25 fragment III (synapsin domain-1) and moderately with fragments IV, V, and VII but not with fragment VI, which codes for the LXXLL motif. The mutation of the LXXLL motif had no effect on CBP interaction, suggesting that Med25 interacts with CBP mainly through the SD-1 and PTOV/ACID regions. Med1/PBP/Trap220 bound exclusively with Med25 fragment 1, coding for the VWA motif.

To summarize, Med25 interacts with the nuclear receptors HNF4 α and RAR via the C-terminal region that encodes the LXXLL motif. Med1, SRC-1, and GRIP-1 interact with Med25 in the N-terminal region. PGC-1 α interacts with Med25 via the C-terminal region but not through the LXXLL motif, while CBP interacts with Med25 through the SD-1 and PTOV domains. To further augment the *in vitro* interaction data, we analyzed these interactions by coexpressing the proteins to determine their localization in HEK293 cells. Med25 was expressed as a green fluorescent protein (GFP) fusion protein and coexpressed with HNF4 α , PGC-1 α , CBP, SRC-1, GRIP-1, and Med1, all of which colocalized in the nucleus, as judged by confocal microscopy (Fig. 1F). NCOA6, shown here as a negative control, did not colocalize with Med25.

Med25 regulates HNF4 α activation. To elucidate the role of Med25 in HNF4 α signaling of the drug-metabolizing enzymes CYP2C9 and CYP3A4, we employed primary human hepatocytes and HepG2 cells (a human liver cell line) and investi-

exogenous ligand). (D to F) *In vivo* interaction of HNF4 α with Med25. (D) Mammalian two-hybrid analysis. *In vivo* interaction analysis on 9XGal4 luciferase promoter expressed in HepG2 cells with the Gal4-HNF4 α ligand binding domain (pBind fusion) and the VP-16 Med25 domains (pACT fusion) confirms the interaction through the LXXLL motif in domain III. Values represent means \pm standard errors (SE) of results from triplicate analyses (*, significantly greater than the level for the control at P values of <0.001). The interaction is abolished by mutating the LXXLL motif to LXXAA. #, significantly lower than the level for domain III ($P < 0.001$). (E) Immunoprecipitation of HNF4 α with Med25 and vice versa. HNF4 α and Flag-Med25, which are absent in HEK293 cells, were expressed ectopically individually and in combination. The nuclear extracts were immunoprecipitated with Flag Ab for Med25 and probed with HNF4 α Ab (top panel); reciprocally, the extracts were immunoprecipitated with HNF4 α Ab and probed with Flag Ab (lower panel). The inputs were from HNF4 α - and Med25-expressed extracts. WB, Western blot. (F) Confocal microscopy. Nuclear receptor HNF4 α and the indicated cofactors are colocalized in the nucleus with GFP-tagged Med25 in HEK293 cells. Endogenous RNA polymerase II is also colocalized with Med25, whereas NCOA6 does not colocalize with Med25 in the nucleus. DAPI, 4',6-diamidino-2-phenylindole.

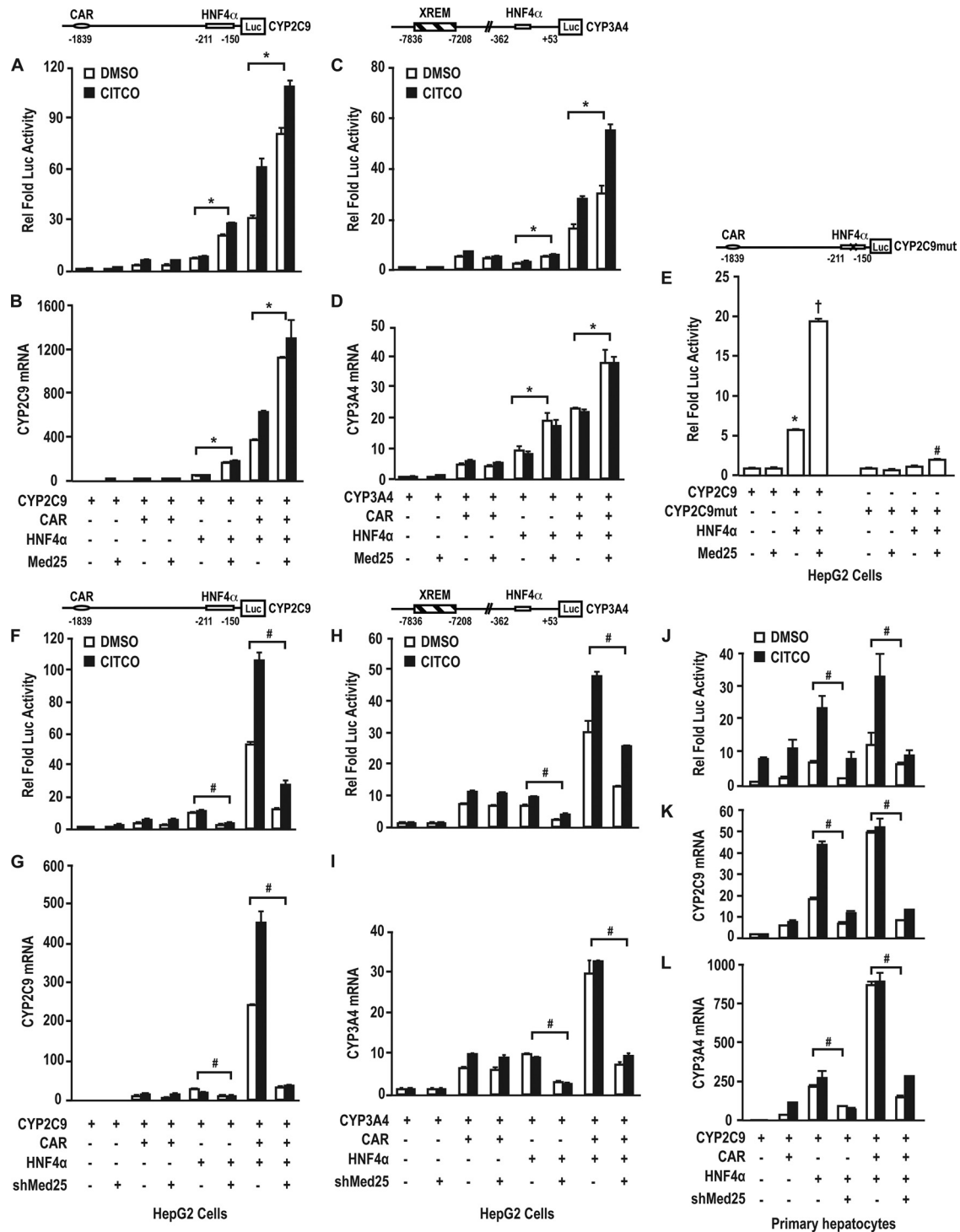


FIG. 2. Med25 regulates HNF4 α -dependent activation. (A) Med25 promoted HNF4 α -dependent *CYP2C9* promoter activation. HepG2 cells were cotransfected with luciferase promoter constructs, Med25, and nuclear receptors (CAR and HNF4 α individually and in combination). Med25 stimulates transcriptional activation by HNF4 α but not CAR. Med25 enhances HNF4 α -dependent *CYP2C9* promoter activity by 3-fold and increases the synergistic activation of *CYP2C9* by CAR and HNF4 α in the presence or absence of the CAR ligand CITCO. Data represent means \pm SE ($n = 3$). *, statistically significant increase between groups indicated by bars ($P < 0.05$). (B) Med25 enhances HNF4 α -induced *CYP2C9* mRNA expression in HepG2 cells as measured by qPCR. *CYP2C9* mRNA is induced by coexpression of Med25 with nuclear receptors (HNF4 α alone or in combination with CAR). Data represent means \pm SE ($n = 3$). *, statistically significant increase between groups indicated by bars ($P < 0.05$). (C) Med25 stimulates HNF4 α -dependent *CYP3A4* promoter activation. HepG2 cells were transfected with a *CYP3A4* luciferase promoter construct along with Med25 and nuclear receptors CAR and HNF4 α . Med25 specifically promotes HNF4 α -dependent but not CAR-dependent *CYP3A4* activation. The synergistic activation of *CYP3A4* by CAR and HNF4 α is also enhanced by Med25. Data represent means \pm SE ($n = 3$). *, statistically significant increase between groups indicated by bars ($P < 0.05$). (D) Med25 enhances HNF4 α -induced *CYP3A4* gene expression

gated the connection between Med25 and the transactivation of HNF4 α with luciferase assays. HepG2 cells were transfected with a 1.9-kb *CYP2C9* promoter construct in the pGL3 reporter vector along with the nuclear receptors CAR, HNF4 α , and Med25 in the combinations shown in Fig. 2A, followed by treatment with the CAR ligand CITCO {6-(4-chlorophenyl)-imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime} (100 nM) or vehicle. We have previously shown that both CAR and HNF4 α activate the *CYP2C9* promoter and that there is a synergistic activation when both receptors are present, which is enhanced in the presence of CITCO (54). As expected, Med25 did not activate the *CYP2C9* promoter, nor did it augment the activation by CAR. However, the activation of the *CYP2C9* promoter by HNF4 α (7) and the synergistic activation by CAR and HNF4 α were markedly enhanced by ectopic expression of Med25. In analogous experiments with *CYP3A4* (Fig. 2C), *CYP3A4* was activated by CAR in the presence and absence of CITCO, but coexpression of Med25 had no further effect. However, HNF4 α also activated the *CYP3A4* promoter, and coexpression of Med25 enhanced this activation. *CYP3A4* was also synergistically activated by CAR and HNF4 α in the presence or absence of CITCO, and the presence of Med25 clearly augmented this activation. Taken together, Med25 enhances HNF4 α -mediated gene activation whether the gene is activated by HNF4 α alone or synergistically with CAR and HNF4 α .

To confirm the activation studies at the gene expression level, we infected HepG2 cells with adenovirus coding for the LacZ, Med25, CAR, and HNF4 α proteins as shown and then analyzed the mRNA levels of *CYP2C9* and *CYP3A4* (Fig. 2B and D). Consistent with the luciferase assay results, CAR and HNF4 α modestly induced the *CYP2C9* mRNA individually. Med25 alone had no effect on *CYP2C9* gene expression, but it enhanced induction by HNF4 α . CAR and HNF4 α synergistically induced *CYP2C9* mRNA, and the induction was in-

creased by the presence of CITCO. Med25 further enhanced this synergistic induction. As shown in Fig. 2D, Med25 alone or in combination with CAR did not induce *CYP3A4* mRNA. However, Med25 increased HNF4 α -dependent *CYP3A4* induction 2-fold and also increased the induction of *CYP3A4* mRNA by CAR and HNF4 α in combination.

To further confirm that Med25 promotes the expression of *CYP2C9* through HNF4 α binding on the promoter, we generated mutation constructs in the HNF4 α binding sites. HepG2 cells were transfected with either the wild-type promoter construct or a construct containing mutations in the three known HNF4 α binding sites, as shown in Fig. 2E. The wild-type *CYP2C9* promoter construct was activated significantly by expression of HNF4 α , and Med25 clearly enhanced this effect. On the other hand, when the HNF4 α response elements in the *CYP2C9* promoter construct were mutated, ectopic expression of HNF4 α had no statistically significant increase in promoter activity. Moreover, the ectopic coexpression of Med25 and HNF4 α failed to activate the mutated promoter, showing that the HNF4 α response elements are necessary for the effect of Med25. Consistent with these observations, the wild-type *CYP2C9* promoter was not activated by an HNF4 α mutant lacking the AF2 domain, nor was HNF4 α activation increased by coexpression with a Med25 construct containing a mutation in the LXXLL domain (see Fig. S3A and B at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). These observations demonstrate that Med25 modulates the expression of xenobiotic gene expression through interaction with a functional HNF4 α protein.

To further examine the regulatory role of Med25 in HNF4 α signaling, we developed adenovirus shRNA constructs to specifically knock down Med25. Out of the three constructs screened, short hairpin Med25 (shMed25) construct II reduced Med25 mRNA expression by more than 60% in HepG2 cells (see Fig. S1A at <http://www.niehs.nih.gov/research/atniehs/labs>

in HepG2 cells. *CYP3A4* mRNA is induced by expression of HNF4 α individually or in combination with CAR. This induction is enhanced by coexpression of Med25. Data represent means \pm SE ($n = 3$). *, statistically significant increase between groups indicated by bars ($P < 0.05$). (E) Mutating the HNF4 α binding sites in the *CYP2C9* promoter abrogates activation by HNF4 α and Med25. The three known proximal HNF4 α response elements in the *CYP2C9* promoter were mutated as previously described (44). HepG2 cells were transfected with the wild-type *CYP2C9* promoter. Expression of HNF4 α significantly transactivates the wild-type *CYP2C9* promoter (*, $P < 0.05$), and cotransfection of Med25 further enhances this transactivation (\dagger , $P < 0.05$). Conversely, there was no statistically significant effect of coexpression of HNF4 α and Med25 on the mutant promoter. #, the level of transactivation of the mutated promoter was significantly lower than that of the wild-type promoter ($P < 0.05$). Data represent means \pm SE, $n = 3$. (F) Silencing Med25 affects *CYP2C9* promoter activation. HepG2 cells were transfected with a *CYP2C9* luciferase promoter construct along with nuclear receptors (CAR and HNF4 α). Med25 was silenced using AdshMed25. Silencing Med25 had no effect on CAR-mediated *CYP2C9* activation but significantly reduced HNF4 α -dependent activation. The synergistic activation of *CYP2C9* was also significantly reduced proportional to the HNF4 α -mediated activation. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (G) Silencing Med25 decreases *CYP2C9* mRNA induction. HepG2 cells were infected with adenovirus containing CAR and HNF4 α individually and in combination for induction of *CYP2C9* mRNA. Silencing Med25 levels does not alter induction by CAR but alters induction by HNF4 α and dramatically reduces the synergistic induction of *CYP2C9* by CAR and HNF4 α . Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (H) Silencing Med25 decreases *CYP3A4* promoter activation by HNF4 α . Reduction of Med25 had no effect on CAR-mediated *CYP3A4* activation but significantly reduces HNF4 α -dependent activation and the synergistic activation of *CYP3A4* by HNF4 α and CAR. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (I) Silencing Med25 decreases *CYP3A4* mRNA induction by HNF4 α . Silencing Med25 had no effect on *CYP3A4* mRNA induction by CAR but decreases induction by HNF4 α and the synergistic induction by CAR and HNF4 α . Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (J) Reduction of Med25 in human primary hepatocytes reduces HNF4 α -dependent *CYP2C9* promoter activation as well as the synergistic activation by HNF4 α and CAR. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (K) Silencing Med25 in human primary hepatocytes reduces *CYP2C9* mRNA induction by HNF4 α (individually and in combination with CAR) in the presence or absence of the CAR ligand CITCO. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (L) Silencing of Med25 in primary human hepatocytes also decreases *CYP3A4* mRNA induction by HNF4 α alone or in combination with CAR in the presence or absence of the CAR ligand CITCO. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$).

/ltp/human/docs/med25supplemental-data.pdf). Reduction of Med25 levels with adenovirus expressing shRNA (AdshRNA) did not affect the endogenous levels of HNF4 α protein (see Fig. S1B, panel 2, at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>); unfortunately, no antibody which will detect endogenous Med25 protein by immunoblotting is available; only ectopic expression of Med25 could be detected by the Flag antibody (see Fig. S1B, panel 1, at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). As shown in Fig. 2F, silencing Med25 did not affect *CYP2C9* promoter activity or its activation by CAR. However, it greatly diminished the transactivation of the *CYP2C9* promoter by HNF4 α and the synergistic activation by CAR and HNF4 α in the presence or absence of CITCO. These results confirm that Med25 is a critical component required for HNF4 α -dependent *CYP2C9* transactivation. Consistent with these observations, when HepG2 cells were infected with (Ad) CAR and/or (Ad) HNF4 α , silencing Med25 diminished HNF4 α -dependent *CYP2C9* mRNA induction and abolished the synergistic induction seen with CAR and HNF4 α , while it did not affect CAR-mediated *CYP2C9* mRNA induction (Fig. 2G).

Silencing Med25 also affected the activation of the *CYP3A4* promoter construct (Fig. 2H). CAR and HNF4 α moderately transactivated *CYP3A4* in the presence and absence of the ligand CITCO. Silencing Med25 had no effect on CAR-mediated transactivation, while HNF4 α -mediated transactivation was diminished. Silencing Med25 also abolished the synergistic transactivation seen with coexpression of CAR-HNF4 α . These luciferase assay results are consistent with *CYP3A4* mRNA induction studies (Fig. 2I), i.e., silencing Med25 affected induction by HNF4 α and the synergistic induction by CAR-HNF4 α but had no effect on induction by CAR.

To extend our observations beyond liver cell lines, we transfected primary human hepatocytes with the *CYP2C9* promoter construct with AdCAR or HNF4 α , individually or in combination (Fig. 2J). *CYP2C9* promoter activity was increased by CITCO. Ectopic expression of CAR slightly enhanced this activation. HNF4 α moderately transactivated *CYP2C9* promoter activity, and the addition of the CAR ligand CITCO enhanced this activation severalfold. This effect of CITCO reflects the translocation of CAR to the nucleus by CITCO (34) and a subsequent synergistic activation between CAR and HNF4 α . Silencing Med25 downregulates the transcriptional activation by HNF4 α as seen with HepG2 cells. Ectopic coexpression of CAR and HNF4 α synergistically activates promoter activity, which is enhanced by CITCO. Even this ectopic nuclear receptor transcriptional activation is abolished by silencing Med25, suggesting that Med25 has a vital status in the formation of the transcriptional complex responsible for promoter activation.

To determine whether this promoter activation is translated into gene expression, primary hepatocytes were infected with Ad scrambled, CAR, HNF4 α , and shMed25 (Fig. 2K), and *CYP2C9* mRNA levels were quantified with qPCR. *CYP2C9* mRNA was induced when cells were treated with CAR and HNF4 α alone or in combination. Treatment with CITCO enhanced induction of *CYP2C9* mRNA by HNF4 α . Coexpression of CAR and HNF4 α further induced *CYP2C9* mRNA; ligand produced no further increase. Silencing Med25 downregulated

CYP2C9 mRNA induction by HNF4 α as well as the CAR-HNF4 α synergistic induction. The induction of *CYP3A4* mRNA mirrored that of *CYP2C9*, although the magnitude of the induction differed (Fig. 2L). To conclude, Med25 plays an important role in the regulation of *CYP2C9* and *CYP3A4* in primary human hepatocytes as well as HepG2 cells, and the reduction of Med25 levels greatly diminishes this regulation.

Med25 is critical for HNF4 α -mediated active transcriptional complex formation. To investigate the molecular mechanisms involved with Med25 in HNF4 α -dependent *CYP* gene regulation, we sought to identify the differential binding of nuclear factors to HNF4 α when Med25 was present or diminished by silencing. HepG2 cells were infected with HNF4 α -Med25 (AdHNF4 α and AdFlag-Med25) or HNF4 α -shMed25 (AdHNF4 α and AdshMed25), and nuclear extracts were prepared. These nuclear extracts were immunoprecipitated with anti-HNF4 α antibody or IgG controls, and the bound proteins were eluted, separated on a 4 to 20% SDS-PAGE gel, and visualized by silver staining as shown in Fig. 3A. Mass spectral analysis of excised gel slices by LC-MS-MS showed that the pattern of transcriptionally important proteins bound to HNF4 α when Med25 was present differed from that found when Med25 was silenced (Fig. 3A, right panel). The complete list of proteins found in both lanes is given in Table S-I at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>. Notably, important proteins that were bound to HNF4 α when Med25 was reduced belonged principally to the transcriptional repression complex anchored by NCOR and MLL4. On the other hand, Med25 expression transformed the HNF4 α complex into a transcriptional activation complex that consists of Med1, Med14, Med8, MLL3, and RNA polymerase II subunits. This suggests that Med25 may play an important role in the association of HNF4 α with the Mediator complex proteins, several other cofactors, and RNA Pol II. To date and to our knowledge, this is the first instance where a single cofactor association has altered the nature of the transcriptional complex of a nuclear receptor.

To confirm the mass spectral data, we immunoblotted the HNF4 α -bound eluates from nuclear extracts in which Med25 was ectopically expressed or silenced (Fig. 3B). IgG was used as the control. HNF4 α was pulled down with HNF4 α antibody from the nuclear extracts of HNF4 α -Med25 and HNF4 α -shMed25. When the immunoprecipitates were probed with anti-Flag antibody, Med25 was detected only in the HNF4 α -Med25 pulldowns, as expected. But when the immunoprecipitates were probed with antibodies for Med8, Med14, MLL3, and RNA polymerase II, these proteins were detected only when the immunoprecipitates were from nuclear extracts of cells infected with HNF4 α -Med25 and not from those infected with HNF4 α -shMed25. Conversely, NCOR was detected only in the eluates from immunoprecipitates from cells infected with HNF4 α -shMed25, not in those from cells infected with HNF4 α -Med25.

To further extend our observations to primary hepatocytes, these cells were infected with scrambled, Flag-Med25, and shMed25 adenovirus and immunoprecipitated with IgG and anti-HNF4 α antibodies as described above. As shown in Fig. 3C, when the eluates were probed with anti-HNF4 α antibodies, HNF4 α was immunoprecipitated by the HNF4 α antibody

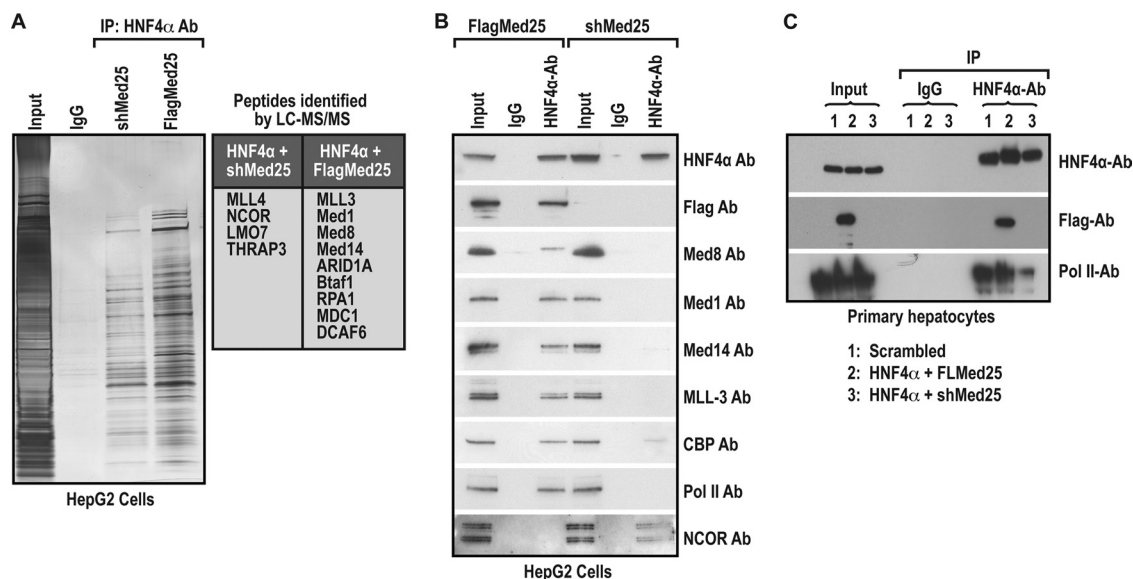


FIG. 3. Silencing Med25 alters the HNF4 α binding complex. (A) Med25 is critical for the HNF4 α binding complex. HepG2 cells were infected with adenoviral vectors expressing the indicated proteins either to increase Med25 (HNF4 α -Flag-MED25) or to reduce Med25 (HNF4 α -shMed25). Nuclear extracts were immunoprecipitated with IgG and HNF4 α antibodies, and the bound proteins were visualized by silver staining. The lanes were excised and subjected to LC-MS-MS identification. Transcriptionally important proteins identified by LC-MS-MS are shown in the table. (B) Immunoblotting of HNF4 α -bound proteins. The above-mentioned nuclear extracts were subjected to immunoprecipitation with HNF4 α and IgG antibodies and probed with the indicated antibodies. The nuclear repressor complex anchored by NCOR is present when Med25 is silenced, while Mediator components, CBP, MLL-3, and RNA polymerase II are identified when Med25 is coexpressed with HNF4 α . (C) Med25 is critical for Pol II association with HNF4 α primary human hepatocytes. Nuclear extracts from primary hepatocytes infected with adenoviral constructs expressing the indicated protein scrambled shRNA (lane 1), HNF4 α -Flag-Med25 (lane 2), and HNF4 α -shMed25 (lane 3) were immunoprecipitated with IgG and HNF4 α antibodies (input shown on the left). The precipitates were probed with the indicated antibodies. RNA polymerase II is found in association with HNF4 α in the presence of Med25 and greatly diminished when Med25 is silenced.

but not by IgG. As expected, Flag was detected only in the immunoprecipitates where Flag-Med25 was ectopically expressed, not in those where Med25 was silenced. Importantly, when the immunoprecipitates were probed with antibody to Pol II, RNA Pol II was detected in association with HNF4 α when Med25 was ectopically expressed, and its association with HNF4 α was highly reduced when Med25 was silenced in primary hepatocytes (Fig. 3C). These proteins were not detected in the IgG controls. Our results suggest that Med25 is critical in controlling the association of the RNA polymerase II complex with the HNF4 α binding complex, irrespective of the cell line, suggesting that this phenomenon is specific to HNF4 α .

Med25 is required for the recruitment of RNA Pol II to the promoter. As described above, we demonstrated with immunoprecipitation that Flag-Med25 associates with HNF4 α and RNA Pol II. In order to demonstrate that this interaction occurs on the promoter, we first employed a modified electromobility supershift assay. Primary human hepatocytes were infected with adenovirus expressing or silencing the indicated proteins. As shown in Fig. 4A, immunoblots of the nuclear extracts confirmed the relative expression levels of HNF4 α , Flag-Med25, GFP, and RNA Pol II. After ectopic expression, the level of HNF4 α was only slightly higher than that observed in the cells infected with the scrambled controls. Reduced levels of Med25 did not change the expression of HNF4 α , GFP, or RNA Pol II.

Nuclear extracts were then incubated with radiolabeled double-stranded oligonucleotides spanning the bp -150 HNF4 α response element of *CYP2C9* in the presence of IgG as the

control. HNF4 α from the nuclear extracts bound to the probe and retarded the mobility of the complex in the gel compared to the level for the free probe. When HNF4 α was ectopically coexpressed with Flag-Med25, the mobility of the complex was slower than that observed for HNF4 α alone (Fig. 4B). The same nuclear extracts were incubated with the anti-HNF4 α antibodies in the presence of labeled oligonucleotide probe (Fig. 4C). HNF4 α antibody supershifted the probe compared to IgG in the samples in which HNF4 α was expressed, i.e., scrambled, HNF4 α , and HNF4 α -shMed25. The complexes seen with Flag-Med25 and HNF4 α -Flag-Med25 were shifted higher than those seen with HNF4 α alone due to their direct interaction. When nuclear extracts were incubated with anti-Flag antibody as shown in Fig. 4D, those containing Flag-Med25 and HNF4 α -Flag-Med25 were supershifted compared to the complexes from scrambled shRNA, HNF4 α , and HNF4 α -shMed25 nuclear extracts. Interestingly, antibody to RNA Pol II (interacting with the endogenous protein) supershifted the labeled probe from the nuclear extracts of scrambled and HNF4 α -treated primary hepatocytes as well as those from Flag-Med25 and HNF4 α -Flag-Med25 hepatocytes. However, when the level of Med25 was reduced (HNF4 α -shMed25) in the nuclear extract, the RNA Pol II antibody did not supershift the complex (Fig. 4E), clearly proving that RNA Pol II does not directly interact with HNF4 α . Rather, its presence in the HNF4 α binding complex is due to its interaction with Med25, which interacts directly with HNF4 α . Med25 is thus responsible for the recruitment or association of RNA Pol II to the HNF4 α binding site *in vitro* or *in vivo*. The interaction of

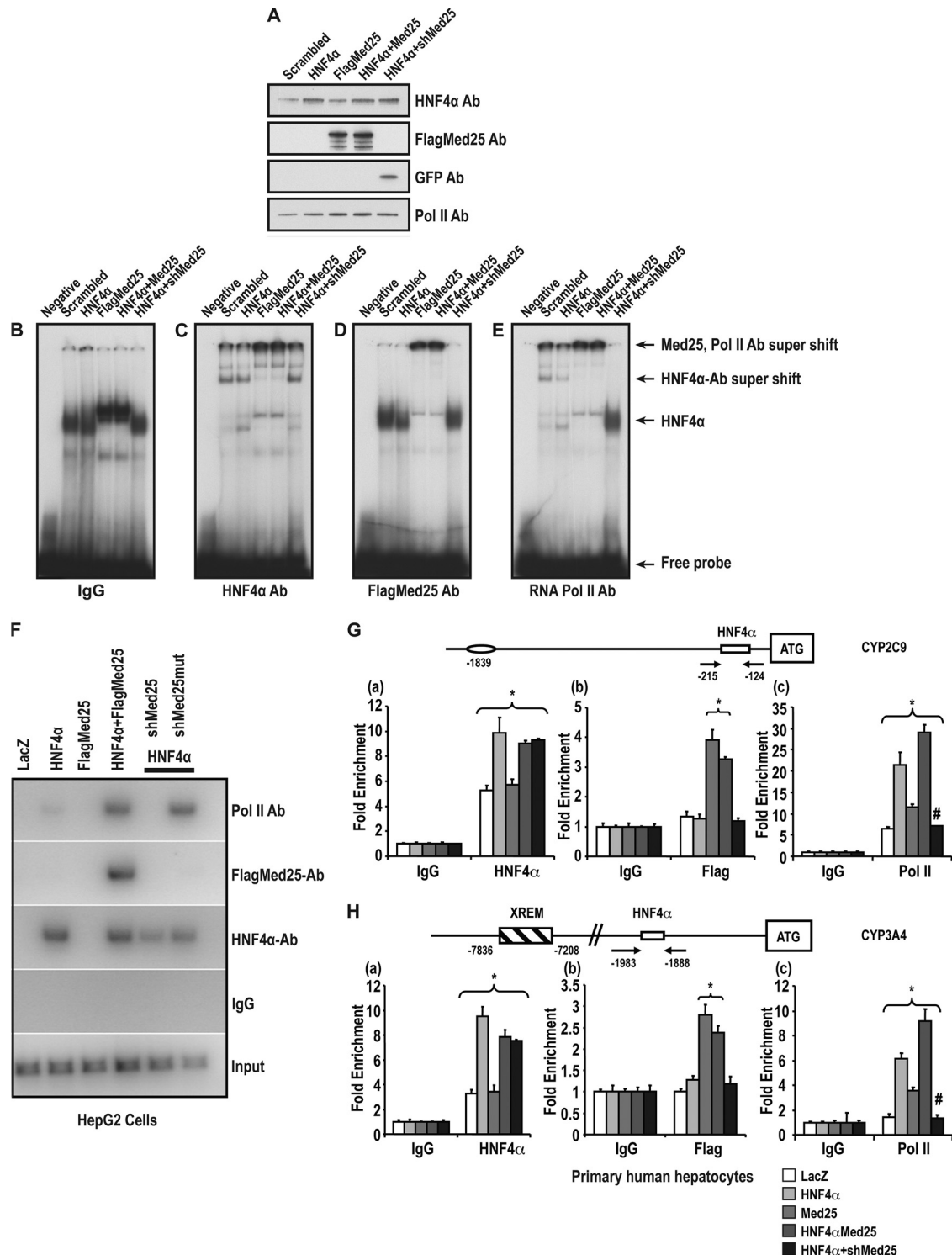


FIG. 4. Med25 is required for recruitment of Pol II to the *CYP2C9* promoter in HepG2 cells and primary human hepatocytes. (A) Relative levels of expression of HNF4 α , Flag-Med25, and Pol II in primary human hepatocytes. Hepatocytes were infected with adenoviruses containing scrambled shRNA, HNF4 α , Flag-Med25, and shMed25 as indicated. The expression levels of the indicated proteins were detected by immunoblot analysis. (B to E) Electrophoretic mobility shift analysis (EMSA) of HNF4 α binding to the bp -150 HNF4 α response element of the *CYP2C9* promoter in primary human hepatocytes. (B) The indicated nuclear extracts were incubated with a 32 P-labeled oligonucleotide containing the proximal HNF4 α response element (at bp -150) of the *CYP2C9* gene in the presence of rabbit IgG. The mixture was separated on a 5% PAGE gel using 0.25 \times Tris-borate-EDTA (TBE). (C to E) The indicated nuclear extracts were incubated with the HNF4 α binding site as before in the presence of antibody to HNF4 α (C), antibody to Flag (D), or antibody to RNA Pol II (E). (F) ChIP analysis of HNF4 α , Med25, and Pol II on the proximal HNF4 α response elements of the *CYP2C9* promoter in HepG2 cells. ChIPs with IgG as a control, HNF4 α , Flag, and Pol II antibodies show the accumulation of the indicated proteins on the HNF4 α binding sites when infected with adenoviruses containing LacZ, HNF4 α ,

HNF4 α -Med25 and its association with RNA Pol II in co-IP and EMSA suggests that Med25 may facilitate the recruitment of Pol II on the *CYP2C9* promoter *in vivo*.

We further analyzed the accumulation of HNF4 α and Med25 on the HNF4 α response element using chromatin immunoprecipitation assays performed with HepG2 cells in a steady state. Although some HNF4 α is expressed endogenously in HepG2 cells, the stringency of our pulldown and washing conditions was such that only ectopically expressed HNF4 α was detected on the HNF4 binding site of *CYP2C9* compared to the levels for the LacZ controls (Fig. 4F). Flag-Med25 was also recruited to the HNF4 α binding site when Flag-Med25 was coexpressed with HNF4 α . Interestingly, a robust recruitment of Pol II was shown on the HNF4 α binding site when HNF4 α and Flag-Med25 were coexpressed. Silencing Med25 prevented the recruitment of RNA Pol II on the promoter. Importantly, a nonfunctional shRNA (shMed25 mut) against Med25 did not affect recruitment of HNF4 α or RNA Pol II to the binding site on the *CYP2C9* promoter, verifying that the association of RNA Pol II to the HNF4 α binding site on the *CYP2C9* promoter is dependent on Med25 expression.

To demonstrate the recruitment of HNF4 α , Med25, and RNA Pol II on the *CYP2C9* promoter in chromatin of primary human hepatocytes, we used quantitative ChIP assays. We infected hepatocytes with adenoviruses expressing LacZ, HNF4 α , Flag-Med25, HNF4 α -Med25, or HNF4 α -shMed25. Chromatin extracts immunoprecipitated with the indicated antibodies were quantified with PCR using SYBR green as shown in Fig. 4G. HNF4 α was recruited to its binding site in all the samples, including endogenous levels as indicated by the LacZ controls. Its recruitment level was slightly higher when HNF4 α was ectopically expressed. Flag antibody exclusively pulled down the HNF4 α binding site when Flag-Med25 was expressed ectopically. RNA Pol II was recruited to the HNF4 α binding site when Med25 was endogenously present or ectopically expressed; however, its recruitment was significantly diminished when Med25 was silenced. A similar recruitment pattern for HNF4 α , Med25, and RNA Pol II was also observed at an HNF4 α site at position -1925 of the *CYP3A4* promoter (Fig. 4H). Taken together, our results show that RNA Pol II recruitment to HNF4 α sites of the promoter region of *CYP2C9* or

CYP3A4 is dependent on the association of Med25 with HNF4 α .

Downregulation of Med25 impairs a specific set of HNF4 α target genes, the cytochrome P-450s, in primary human hepatocytes. To elucidate the regulatory role of Med25 in HNF4 α signaling in the liver, we infected primary human hepatocytes (Fig. 5A to G) and HepG2 cells (see Fig. S4A to F at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>) with adenoviruses for the ectopic expression of CAR and HNF4 α and simultaneous knockdown of Med25; we then treated the cells with the CAR ligand CITCO. As shown in Fig. 5, *CYP2C9*, *CYP2B6*, *CYP1A2*, and *CYP2D6* mRNAs were upregulated by HNF4 α in primary hepatocytes and silencing Med25 decreased this upregulation. *CYP2C9* and *CYP2B6* were further induced by coexpression of CAR, and *CYP2C9* induction was increased by the presence of CITCO. Furthermore, when Med25 was silenced, the induction of the *CYP2C9* and *CYP2B6* genes by HNF4 α or HNF4 α plus CAR was significantly decreased. In contrast, HNF4 α target genes involved in glucose homeostasis (glucose-6-phosphatase [G6PC]), phosphoenolpyruvate carboxykinase 1 (PCK1), and fatty acid binding protein (FABP4; ap2) were not affected by silencing the Med25, as shown in Fig. 5E to G. Similar results were seen in HepG2 cells, as shown at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>.

Overall, these results suggest that the association of Med25 does not affect all HNF4 α targets but that a specific subset of target genes is upregulated by ectopic expression of Med25 and downregulated by silencing Med25. To our knowledge, this is the first report of a cofactor that specifically regulates the expression of a subset of genes, in this case the cytochrome P-450s.

Reduction of Med25 alters specific HNF4 α -signaling pathways. HNF4 α is an important regulator of various metabolic parameters in human liver. We have shown herein that Med25 has a vital link as a coactivator of HNF4 α . To examine the functions of Med25 in various gene partnerships, we reduced the expression of Med25 in primary human hepatocytes by adenoviral shRNA targeted against Med25. For comparison, we reduced HNF4 α by adenoviral shRNA and ectopically overexpressed HNF4 α . Reduction of endogenous Med25 had

Flag-Med25, HNF4 α -Flag-Med25, HNF4 α -shMed25, or HNF4 α -shMed25mut as shown. Silencing Med25 does not prevent recruitment of HNF4 α protein to the HNF4 α site but prevents recruitment of RNA polymerase II to this site. An ineffective shRNA construct (shMed25mut) that fails to reduce the expression of Med25 does not prevent recruitment of RNA polymerase II to the HNF4 α binding site. Flag antibody detects the accumulation of Med25 to this site when Flag-Med25 and HNF4 α are ectopically expressed. (G) Accumulation of HNF4 α , Med25, and RNA Pol II on the *CYP2C9* promoter in primary human hepatocytes. Chromatin extracts from primary human hepatocytes infected with adenovirus expressing either LacZ, HNF4 α , Med25, HNF4 α -Med25, or HNF4 α -shMed25 were subjected to ChIP analysis with anti-HNF4 α (a), Flag (b), and Pol II (c) antibodies and IgG as a control. The immunoprecipitates were analyzed by qPCR with primers flanking the HNF4 α binding region in the promoter of *CYP2C9*. Data shown are representative of three independent experiments. *, significant enrichment of HNF4 α on the *CYP2C9* promoter indicated by bars ($P < 0.05$); #, enrichment of HNF4 α on the *CYP2C9* promoter is significantly decreased when Med25 is silenced (HNF4 α versus HNF4 α -shMed25; $P < 0.05$). (H) Accumulation of HNF4 α , Med25, and RNA Pol II on an HNF4 α site at kb -1.9 of the *CYP3A4* promoter in primary human hepatocytes. Chromatin extracts from primary human hepatocytes infected with adenovirus expressing either LacZ, HNF4 α , Med25, HNF4 α -Med25, or HNF4 α -shMed25 were subjected to ChIP analysis with anti-HNF4 α (a), Flag (b), and Pol II (c) antibodies and IgG as control. The immunoprecipitates processed as before were analyzed by qPCR with primers flanking the HNF4 α binding region (1.9 kb) in the promoter of *CYP3A4*. Data shown are representative of three independent experiments. *, significant enrichment of HNF4 α on the *CYP3A4* promoter indicated by bars ($P < 0.05$); #, enrichment of HNF4 α is significantly decreased when Med25 is silenced (HNF4 α versus HNF4 α -shMed25; $P < 0.05$).

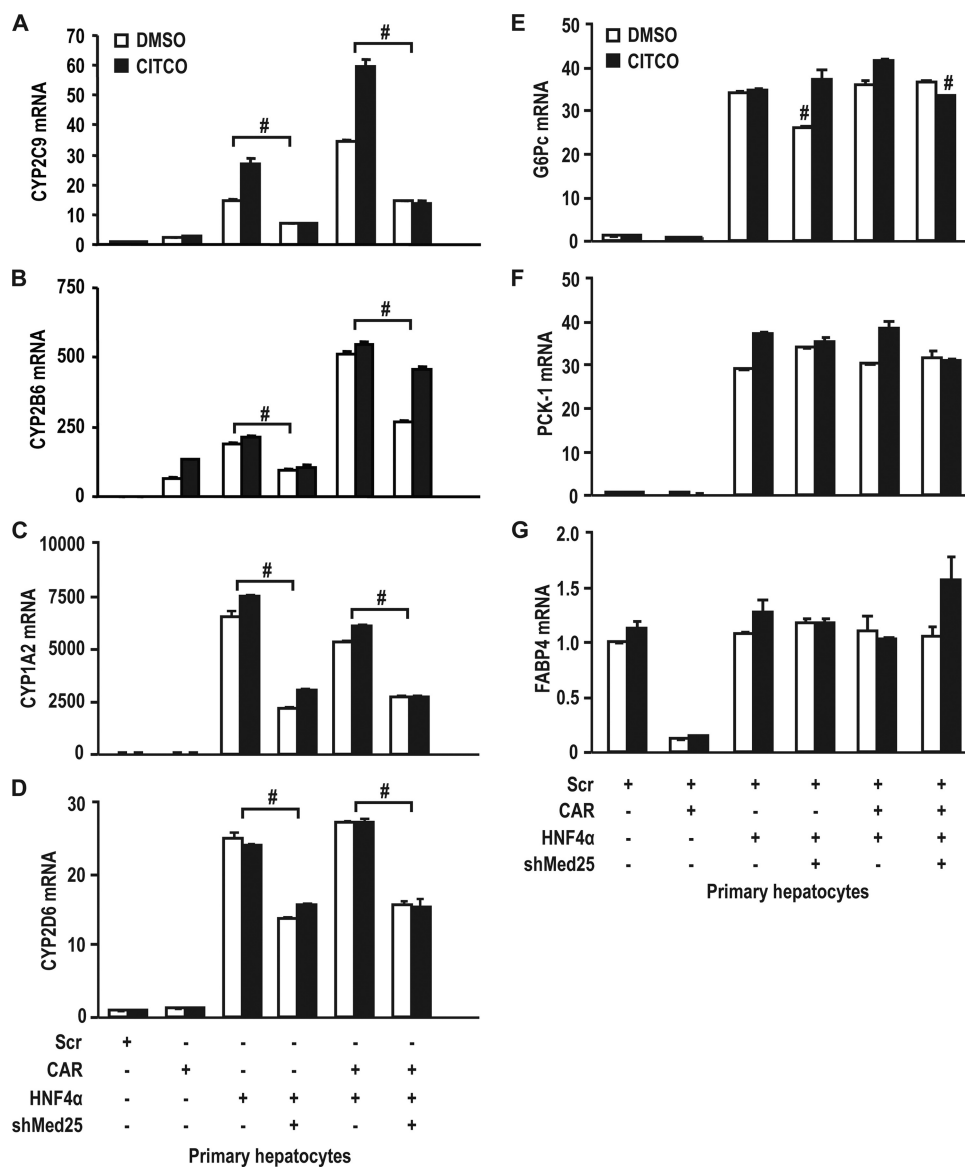


FIG. 5. Med25 regulates expression of specific xenobiotic metabolizing enzymes in primary human hepatocytes. Primary hepatocytes were infected with adenovirus coding for ectopic expression of CAR, HNF4 α , and Med25, and expression was reduced with adenoviral shRNA. (A) Silencing Med25 downregulates the induction of CYP2C9 mRNA by HNF4 α or HNF4-CAR in primary hepatocytes. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (B) The induction of CYP2B6 mRNA by HNF4 or HNF4 α and CAR is downregulated by silencing Med25. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (C) Silencing Med25 inhibits CYP1A2 mRNA induction by HNF4 α . CYP1A2 is induced by HNF4 α but is not activated by CAR. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). (D) Silencing Med25 affects CYP2D6 mRNA induction by HNF4 α . The upregulation of CYP2D6 by HNF4 α is reduced by silencing Med25. Data represent means \pm SE ($n = 3$). #, significant decrease between groups indicated by bars ($P < 0.05$). The classical targets of HNF4 α which are involved in glucose homeostasis and fatty acid binding are not affected by the loss of function of Med25. (E) G6Pc, a direct HNF4 α target in human liver, is not affected by silencing Med25. Data represent means \pm SE ($n = 3$). #, shMed25 modestly but significantly reduces HNF4 α induction ($P < 0.05$). (F) PCK1 is also a direct target of HNF4 α in human liver but is not affected by silencing Med25. (G) FABP4 (ap2) is not induced by the expression of HNF4 α in primary hepatocytes and is not affected by the loss of Med25 function. CAR downregulates FABP4 gene expression.

no effect on primary hepatocyte viability or the expression of HNF4 α mRNA (see Fig. S5 at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). Microarray analysis and ingenuity pathway analysis (IPA) identified two main pathways that were affected by the reduction of endogenous Med25: (i) the xenobiotic signaling

pathway, affecting drug metabolism (Fig. 6A) ($P = 3.43 \times 10^{-4}$), and (ii) the fatty acid oxidation pathway, affecting lipid metabolism (see Fig. S6A at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>) ($P = 4.25 \times 10^{-6}$). Since HNF4 α target genes are found under a variety of pathophysiological conditions, we focused on all the

CYP genes, the majority of which are regulated by HNF4 α and involved in xenobiotic metabolism. Reduced levels of Med25 were found to downregulate 37 of the total 52 CYP genes, including *CYP2C9*, *CYP3A4*, and *CYP2B6*, while 15 genes were found to be upregulated by the reduced expression of Med25 (Fig. 6B; see also Table S-II at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). In comparison, the majority of the CYP genes that were downregulated by silencing Med25 in primary hepatocytes were also upregulated ectopic expression of HNF4 α . Ingenuity pathway analysis showed that the HNF4 α -driven network involving cellular assembly, organization, function maintenance, and transport is the topmost network affected by the reduced levels of Med25 (Fig. 6C).

IPA analysis identified PPAR α signaling as one of the major pathways affected by the reduced levels of Med25. Genes involved in fatty acid metabolism (such as those involved in oxidation, ketone body synthesis, fatty acid binding, transport, and activation) were analyzed; microarray analysis revealed that 17 of these genes were downregulated by reduced expression of Med25. One of the noted genes, the PPAR α gene, was downregulated, which drives fatty acid metabolism (see Fig. S6A and B and Table S-III at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). qPCR confirmed the decreased expression of PPAR α and several of its target genes, including those encoding acyl-CoA carboxylase (*Acox*), liver bifunctional enzyme (*Ehhadh*), medium-chain acyl-CoA dehydrogenase (*MCAD*), *SPOT14*, and *CYP4AF2*, while *CYP4A11* and the fatty acid transporter (*CD36*) were upregulated by reduced Med25 levels (Fig. 6D to K).

Reduced levels of Med25 impair drug metabolism in primary human hepatocytes. Activation of the xenobiotic sensing nuclear receptors (CAR/PXR) induces drug metabolism in the liver. The regulation of HNF4 α by Med25 suggests that reduced availability of Med25 could impair xenobiotic signaling and the metabolism of drugs *in vivo*. Indeed, in primary human hepatocytes, when Med25 was blunted with shRNA (targeted against Med25) (Fig. 7A), basal levels of CYP2C9-mediated metabolism were decreased by 30% compared to the level for the control (primary human hepatocytes infected with LacZ), and CYP3A4/5 metabolism was reduced by 50%. Metabolism of a CYP2C8 substrate, on the other hand, was not affected by reduced availability of Med25. When HNF4 α levels were elevated by ectopic expression of HNF4 α and Med25 expression was reduced by shRNA, CYP2C9-mediated metabolism was reduced by 30% compared to the level for hepatocytes with only elevated HNF4 α . Metabolism of a CYP2C8-specific substrate remained unchanged. Metabolism of testosterone, a substrate for the CYP3A4/CYP3A5 enzymes, was surprisingly decreased by ectopic expression of HNF4 α and unchanged by simultaneously silencing Med25. However, testosterone is a shared substrate for the two enzymes, which complicates the interpretation of this data. Our data suggest that CYP2C9 expression and substrate metabolism are tightly regulated by Med25 and provide evidence for the involvement of Med25 in CYP3A4 expression. CYP2C8, on the other hand, is regulated by HNF4 α , but Med25 appears to have no role in the expression and substrate metabolism of CYP2C8.

DISCUSSION

The liver is the primary organ that responds to a variety of stimuli to regulate drug and lipid metabolism, including detoxification, storage, transport, and elimination (46). Hepatocyte nuclear factor 4 α is implicated in regulating the genes involved in metabolic processes (22). Dysfunctional signaling in the HNF4 α network is linked to several metabolic disorders, including maturity-onset diabetes of the young (MODY) (20), diabetes, and atherosclerosis, and to altered drug metabolism and clearance (6, 13, 16). Several coactivators belonging to different subcomplexes have been discovered and implicated in gene regulation controlling the various metabolic processes, either directly interacting with HNF4 α (47) or interacting in tandem with other nuclear receptors (CAR, GR, and C/EBP) (40, 59, 60). While many of these cofactors were found to bind directly or indirectly to nuclear receptors to form the transcriptional complex, how they are targeted to specific loci on chromatin to give the regio- and genospecificity remains poorly understood. In the present study, we have identified Med25 as an interacting partner of HNF4 α that transforms the inactive transcriptional complex to an active transcriptional complex and uncovered a role for Med25 in drug and lipid metabolism in the human liver. Med25 is variably associated with the Mediator complex (36, 50). Ectopic expression of HNF4 α and Med25 stimulates many drug metabolism pathways, while the reduction of Med25 decreases them, clearly linking Med25 to the HNF4 α -signaling network.

Mediators are a complex of coactivators anchored by Med1 (28) that are able to synchronize gene expression to a variety of environmental signals regulating diverse biological processes (55). However, evidence suggests that not all NR-mediated transcriptional activity is dependent on Med1 (35), and transcriptional complexes could be formed in the absence of Med1 (18, 32). In our quest to identify other cofactors that regulate HNF4 α signaling, we have identified Med25. Interestingly, although many cofactors bind with all nuclear receptors, Med25 is an exception since it fails to bind with two xenobiotic sensing receptors, CAR and PXR, or the vitamin D receptor (32).

Several known coactivators have been identified in the immunoprecipitates of HNF4 α belonging to the CBP-HAT complex (CBP and SRC-1), the Mediator complex (Med8), and the ASCOM complex (NCOA6), in addition to PGC-1 α and RNA Pol II. We have now identified Flag-Med25 and Med8 as additional coactivators. Interestingly, most of these coactivators were found to bind directly with Med25 in GST pulldown *in vitro* interaction assays, but the sites of interaction on Med25 are quite different. HNF4 α binds to Med25 through the LXXLL motif, whereas chromatin modifiers such as CBP interact with the middle of the Med25-spanning PTOV domain. PGC-1 α interacts with the C-terminal region but not through the LXXLL motif. Med1, SRC-1, and GRIP interact in the N-terminal region (see Fig. S2C, diagram at bottom, at <http://www.niehs.nih.gov/research/atniehs/labs/ltp/human/docs/med25supplemental-data.pdf>). Although NCOA6 was identified in the HNF4 α complex, it does not bind with Med25 directly. HNF4 α and the majority of these coactivators colocalize with Med25 in the nucleus, whereas NCOA6 does not colocalize, suggesting that although these two proteins belong

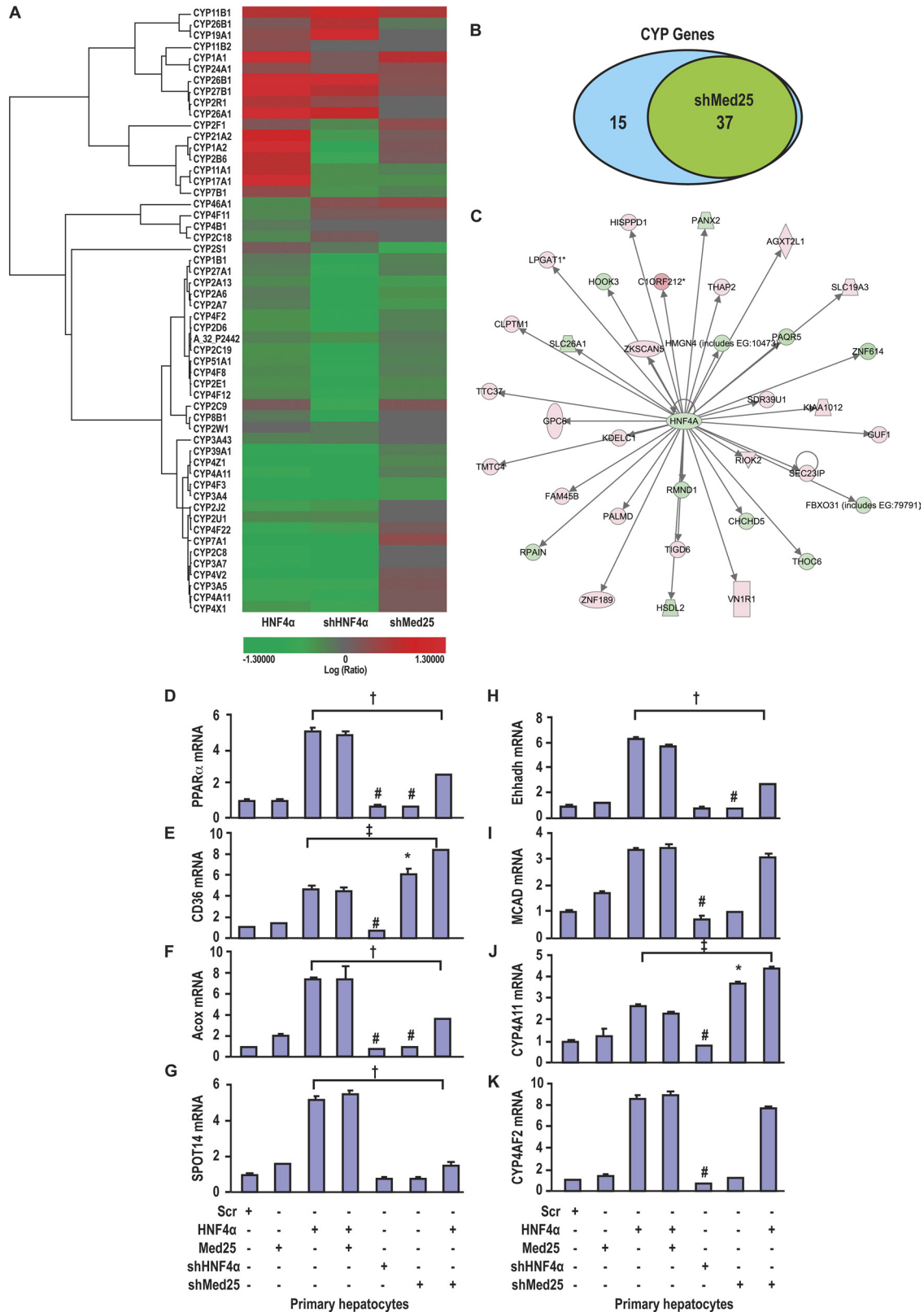


FIG. 6. Downregulation of Med25 impairs a specific set of HNF4 α target genes in human primary hepatocytes. (A) Microarray analysis of *CYP* genes from scrambled-virus (control) and Med25 knockdown in primary human hepatocytes. The log ratios of shMed25/scrambled, shHNF4 α /scrambled, and HNF4 α /scrambled are presented in the heat map. (B) Venn diagram representation of the subset of *CYP* genes involved in drug metabolism that were reduced in the primary human hepatocytes treated with shMed25. (C) Schematic diagram of the HNF4 α network that was significantly affected by the reduced levels of Med25 in primary human hepatocytes as identified in IPA analysis. Green denotes downregulated

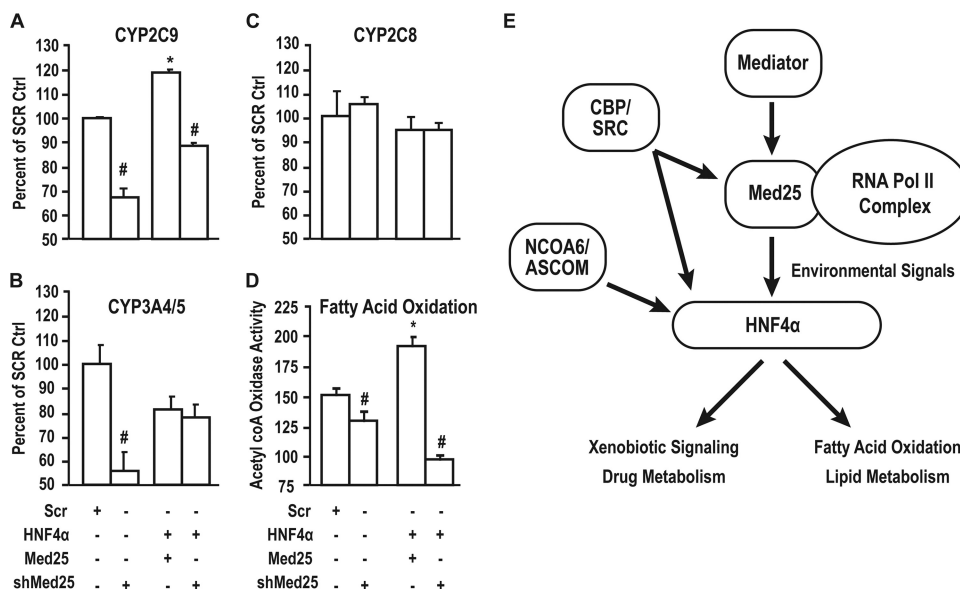


FIG. 7. Reduction of Med25 in primary human hepatocytes alters drug metabolism. (A to C) Regulation of the metabolism of diclofenac (CYP2C9); paclitaxel (CYP2C8), and testosterone (CYP3A4/5) in microsomes from primary human hepatocytes treated with the indicated adenoviruses. Data represent means \pm SE ($n = 3$). *, silencing Med25 significantly increased enzymatic activity compared to the level for the control (scrambled) ($P < 0.05$); #, significantly decreased enzymatic activity compared to the level for the control (scrambled) ($P < 0.05$). (D) Reduction in Med25 levels affects fatty acid oxidation. The oxidation rate of palmitic acid in primary human hepatocytes was reduced significantly when Med25 levels were reduced compared to the levels for primary human hepatocytes treated with scrambled virus. Data represent means \pm SE ($n = 3$). *, significantly increased enzymatic activity compared to the level for the control (scrambled) ($P < 0.05$); #, significantly decreased enzymatic activity compared to the level for the control (scrambled) ($P < 0.05$). (E) Schematic model of the Med25 regulation of HNF4 α target genes in drug and lipid metabolism and their associated subcomplexes, which interact directly with either the promoter or Med25 to execute the finely tuned gene regulation on specific environmental clues.

to the HNF4 α -bound complex, they may exist as separate sub-complexes or be recruited in a sequential order.

Med25 enhances the transactivation potential of HNF4 α . However, the synergistic activation of two target genes, CYP2C9 and CYP3A4, by CAR and HNF4 α is further boosted by expression of Med25. The CAR ligand CITCO enhanced the synergistic transcriptional effect, suggesting that Med25 might be the final piece of the puzzle explaining the transcriptional activation of these target genes by xenobiotics.

The effect of Med25 appears to be solely mediated through a functional HNF4 α protein, and reduction of Med25 levels inhibited the HNF4 α -dependent activation of CYP2C9 but not CAR-mediated transcriptional activity. The synergistic activity of CAR and HNF4 α was also blunted when endogenous Med25 levels were reduced, as seen from CYP2C9 and CYP3A4 promoter activation assays and mRNA induction assays. Our studies show that Med25 not only is essential for mediating HNF4 α -dependent upregulation of target genes but also dramatically influences CAR-dependent upregulation through HNF4 α (Fig. 2). These transcriptional effects were seen in HepG2 cells and in primary hepatocytes, suggesting

that the Med25 effects are not cell line specific and are thus applicable to human liver drug metabolism.

After establishing Med25 as a coactivator of HNF4 α , we explored the purpose of Med25 in the transcriptional complex. On immunoprecipitation with HNF4 α , nuclear extracts depleted of Med25 bound a different transcriptional complex than when Med25 was ectopically expressed. Mass spectral identification of all the proteins indicated that Med25 depletion resulted in a transcriptional complex, as indicated by the presence of NCOR and MLL4, whereas in the presence of Med25, we observed a transcriptional complex with coactivators such as Med1, Med8, Med14, MLL3, and RPA-1. We suggest that Med25 is essential for converting the NCOR-bound HNF4 α complex (inactive) to the Mediator-bound HNF4 α transcriptional (active) complex. At the molecular level, although it has been speculated that the Med1-anchored Mediator complex was required for the recruitment of RNA polymerase II, EMSA and ChIP data from HepG2 cells and primary human hepatocytes clearly showed that for a subset of genes typified by CYP2C9 and CYP3A4, it is Med25 and not a Med1-anchored Mediator complex that is essential for Pol II

genes, and pink and red denote upregulated genes. (D to K) qPCR analysis of mRNA of selected genes from fatty acid oxidation pathways from total RNA of primary human hepatocytes infected with the indicated adenovirus. Data represent means \pm SE ($n = 3$). #, significantly decreased mRNA expression compared to the level for the control (scrambled shRNA) ($P < 0.05$); †, significantly decreased mRNA expression in HNF4 α -shMed25 samples compared to the level for HNF4 α ($P < 0.05$); *, shMed25 significantly increased mRNA expression compared to the level for the control (scrambled) ($P < 0.05$); ‡, the level of mRNA expression was significantly higher in cells infected with HNF4 α -shMed25 than in those expressing ectopic HNF4 α alone ($P < 0.05$).

recruitment to the target promoter. Given the fact that Med25 is not part of the Mediator complex but an associated protein, these data clearly show that Med25 is essential for the recruitment. Moreover, from qPCR analysis we found that when Med25 was depleted, PPAR α levels were decreased, whereas when HNF4 α levels were increased, PPAR α levels increased. In addition, PPAR α would also require Med25 for transcription regulation of its target genes, namely, those encoding Acox, Ehhadh, etc. These results suggest that PPAR α -mediated fatty acid oxidation may be regulated by Med25, leading to alterations in lipid metabolism, one of the two pathways identified in the IPA analysis. The effects of a reduction in Med25 only partially parallel those of a reduction in HNF4 α . Clearly, as the Venn diagram indicates, there is only a partial overlap between the genes that are upregulated by overexpressing HNF4 α and those that are downregulated by silencing Med25.

Interestingly, the expression of HNF4 α target genes belonging to pathways such as Pck1 and G6PC involved in glucose homeostasis was not affected by reduced availability of Med25. These results suggest that Med25 acts in concert with HNF4 α in regulating only genes involved in specific pathways. In the future, it will be of interest to investigate why Med25 regulates only some genes containing HNF4 α binding sites in the promoter region. For the genes that were selected for further analysis by qPCR, we note that an HNF4 α response element was quite close to the transcription start site, whereas in the nonresponsive genes the binding site was far from the start site. In the future, an unbiased genome-wide bioinformatics approach could help identify the mechanism that determines the selectivity of Med25 in regulating only certain HNF4 α -dependent pathways.

In summary, we have shown that Med25 plays an important role in the regulation of drug and lipid metabolism in primary human hepatocytes as well as in human liver cell lines. While there are many known coactivators described for modulating a plethora of signaling pathways, we show that Med25 confers selectivity and plays a vital role in the recruitment of various other cofactors through HNF4 α binding sites to mediate the effects of HNF4 α on metabolism of drugs and lipids.

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We declare that we have no conflict of interest.

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